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Augmented Latent Membrane Protein 1 Expression from Epstein-Barr Virus Episomes with Minimal Terminal Repeats[▽]

Allison M. Repic,[1,2] Mingxia Shi,[1,2] Rona S. Scott,[1,2,3] and John W. Sixbey[1,2,3]*

Center for Molecular and Tumor Virology,[1] Department of Microbiology and Immunology,[2] and Feist-Weiller Cancer Center,[3] Louisiana State University Health Sciences Center, Shreveport, Louisiana

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The major oncogene of the Epstein-Barr virus (EBV), latent membrane protein 1 (LMP1), can be expressed from either of two promoters, ED-L1 or L1-TR, producing mRNAs of 2.8 kb or 3.5 kb, respectively. L1-TR, active in nasopharyngeal carcinoma and Hodgkin's lymphoma, is located within the first of a highly variable reiteration of terminal repeat (TR) sequences that are joined by random recombination upon circularization of the linear genome at entry into cells. To determine whether the resultant TR number affects LMP1 promoter activity, we isolated single-cell clones bearing episomes of distinct TR numbers (6TR to 12TR) from epithelial cells newly infected with EBV. LMP1 mRNA levels correlated directly with the quantity of LMP1 protein expressed but varied inversely to TR number. Unexpectedly, the 3.5-kb transcript predominated only at lower TR reiterations. Diminished L1-TR activity in the context of a higher TR count was confirmed with a green fluorescent protein (GFP) reporter construct driven by L1-TR. Various levels of LMP1, expressed from virus isogenic in all but TR number, produced divergent morphological and growth phenotypes in each cell clone. Abundant LMP1 in 6TR cells yielded a relatively cytostatic state compared to the proliferative one produced by intermediate and smaller amounts in 8TR and 12TR clones. These findings suggest that the diversification of TR number, inherent in a round of EBV reactivation and reinfection, may itself be a component of the oncogenic process. The replicative burst preceding onset of many EBV-linked cancers may increase the likelihood that LMP1 levels compatible with clonal outgrowth are achieved in a subset of infected cells.

The Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1), a key regulator of EBV-induced cell proliferation, mimics the CD40 receptor but operates in a ligand-independent manner, stimulating multiple signaling pathways that activate NF-κB, AP-1, inhibitor-of-differentiation (Id1 and Id3), and STAT-mediated transcription (16, 23, 24, 31, 35). LMP1 has opposing activities on cell growth, producing inhibitory effects at the physiologically high end of expression in contrast to proliferative phenotypes at intermediate levels (10, 11, 15, 20, 25, 29, 33). Such contrasting outcomes, when viewed in terms of the natural variation in expression up to 100-fold among clonal populations of B lymphocytes (33), make elucidation of mechanisms of LMP1 regulation a central issue in understanding the biology of EBV.

Upon entry into cells, the linear EBV genome forms a covalently closed circle via its terminal repeats (TRs), bringing regulatory regions of latency genes at either end of the linear genome into a contiguous DNA segment. LMP1 is expressed from either of two promoters ~600 bp apart, whose relative activity varies by cell type infected (Fig. 1A). Both transcripts use the same initiator codon, thereby encoding an identical protein. In B lymphocytes, LMP1 is encoded predominantly as a 2.8-kb mRNA, which initiates from a site (ED-L1 promoter) that requires transactivation by the Epstein-Barr virus nuclear antigen 2 (EBNA2) (28). ED-L1 can also be activated independently of EBNA2 by cellular factors, such as the STATs, IRF7, and the activating transcription factor 4 (ATF4), a component of the PERK signaling pathway (8, 34, 44, 49). In addition, the EBNA1-dependent transcriptional enhancer FR located within the viral origin of plasmid replication *oriP* (Fig. 1A) acts across fused TRs to increase expression of LMP1 as much as 200-fold (21, 46).

In epithelial cells, on the other hand, where EBNA2 is not expressed, transcription of a 3.5-kb LMP1 mRNA can initiate from L1-TR, a TATA-less promoter situated in the first terminal repeat (7, 22, 47). Initiation of transcription occurs only from within the first repeat, not the reiterated and more distal TR units, suggesting that unique sequences adjacent to the first repeat may also contribute to L1-TR activity (47). Although regulation of L1-TR is less well studied by comparison, cellular factors Sp1 and STAT3 contribute to activity of this promoter (8, 47, 54), as does the endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR) protein XBP-1 (27).

The proximity of both LMP1 promoters and the FR enhancer to TR sequences suggests scenarios whereby the variable reiteration of TRs may figure into the complexity of LMP1 regulation. For example, as the distance between promoters and enhancer is lengthened by intervening repetitive 538-bp units (Fig. 1A) that may differ by as many as 20 repetitions in latently infected cells from an individual donor (5), is activity of the FR enhancer compromised (3)? Does the position of the L1-TR promoter within GC-rich repeats make it more vulnerable to silencing by methylation, or do inactive promoter sites in distal TRs vie for transcription factors? Here, we have determined an inverse relationship between LMP1 levels and TR number in epithelial cell culture, with increased activity of the L1-TR promoter at lower TR reiterations.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, LSU Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130. Phone: (318) 675-4272. Fax: (318) 675-5764. E-mail: jsixbe@lsuhsc.edu.

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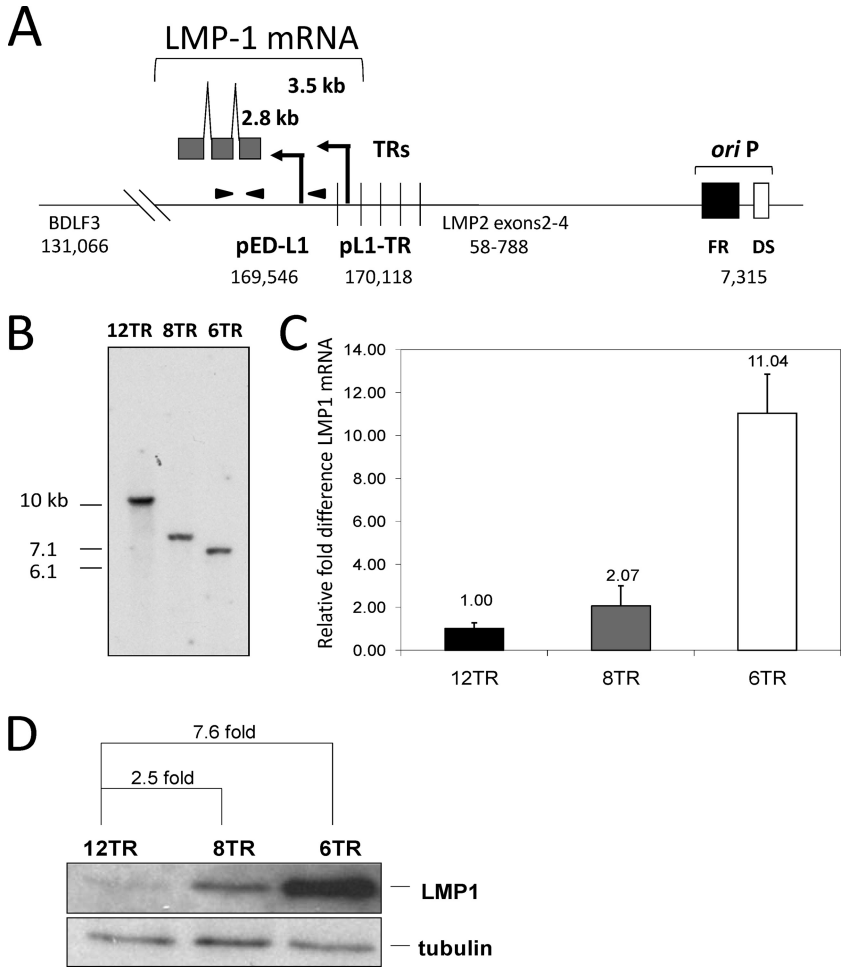

FIG. 1. Effect of EBV terminal repeat number on levels of LMP1 expression. (A) Schematic of fused TRs (vertical lines) after circularization of the EBV genome in relation to LMP1 regulatory elements. Numerals are EBV coordinates for B95-8 (2) (NCBI GenBank accession number VO1555). Bent arrows indicate LMP1 promoters; the black box is the EBNA1-dependent enhancer (FR, family of repeats) in the origin of plasmid replication (*oriP*). Gray boxes represent exons 1 to 3 of LMP1. Arrowheads indicate location of PCR primers used to distinguish 3.5-kb LMP1 transcript from total LMP1 mRNA. Insertion sites of antibiotic-resistant cassettes in EBV strains Akata and B95-8 are located in BDLF3 and LMP2 exons 2 to 4, respectively. (B) Southern blot of BamHI-digested cellular DNA from three CCL20.2 epithelial cell clones infected with B95-8 recombinant EBfaV-GFP that was hybridized to a NJhet (TR-specific) probe. Terminal repeat number (indicated at top) was calculated from molecular weight of band minus size of unique sequences at either end of TRs encompassed by BamHI restriction sites divided by size of one TR unit. Note that the EGFP-Neo^r cassette in recombinant B95-8 introduced a novel BamHI restriction site, thereby reducing the quantity of unique EBV DNA normally contained in the like restriction fragment of wild-type EBV from ~7.5 kb to 4.1 kb (45, 51). Molecular size markers are indicated on the left. (C) Relative fold difference in LMP1 mRNA as determined by RQ RT-PCR. Each cell clone is designated by its distinct episomal TR number. Shown are values from four independent experiments, with error bars representing the standard error of the mean (SEM). (D) Immunoblot of LMP1 protein in infected cell clones. Numerical values represent fold difference in LMP1 expression relative to cell clone indicated after normalization to tubulin loading control, as determined by densitometry.

MATERIALS AND METHODS

Epithelial cell culture and recombinant EBV strains. CCL20.2 (American Type Culture Collection) is a carcinoma cell line that was stably transfected with plasmids expressing the EBV B-cell receptor CR2 and the human class II transactivator CIITA that induces expression of EBV coreceptor major histocompatibility complex (MHC) class II. CCL20.2-CR2-CIITA cells were infected with virus preparations of EBfaV-GFP, a B95-8 recombinant in which the enhanced GFP (EGFP)/neomycin resistance (Neo^r) cassette was inserted in exons 2, 3, and 4 of the LMP2 gene locus, effectively eliminating LMP2 expression (gift of R. Longnecker, Northwestern University) (39, 51). Because virus preparations from the B95-8-derived cell line contain mixed populations of recombinant (LMP2$^-$ EGFP$^+$) and wild-type EBV, low multiplicities of infection were used to infect cells. After G-418 (Mediatech) selection, single-cell clones were derived by limiting dilution and then screened for the presence of the recombinant B95-8 genome alone, on the basis of a positive PCR for EBV gene segment BHRF1 and the inability to amplify LMP2-encoding regions. CCL20.2/B95-8 clones were further characterized with respect to episomal copy number, size of fused terminal repeat sequences, and pattern of latent gene expression (EBNA2, LMP2A, and LMP1). The CCL185 lung carcinoma cell line (ATCC; A549) was infected with virus preparations of EBVneo^r, a recombinant virus generated by insertion of a neomycin resistance cassette in the BDLF3 open reading frame of EBV strain Akata (gift of L. Hutt-Fletcher; LSU Health Sciences Center—Shreveport) (4). CCL185/Akata clones were derived as described above and characterized with respect to the episomal copy number, size of fused terminal repeat sequences, and pattern of latent gene expression. Epithelial cells were cultured in Dulbecco's modified Eagle's media (DMEM; Mediatech) supplemented with 10% fetal bovine serum (HyClone), glutamine, and penicillin-streptomycin.

EBV copy number and TR quantification in cell clones. The EBV DNA copy number in cellular clones was determined by real-time quantitative PCR (RQ PCR) using the TaqMan fluorogenic system (PE Applied Biosystems) with

primers and probes that targeted the single-copy BamHI K fragment of EBV DNA (primers 5′-GGATGCGATTAAGGACCTTGTT-3′ and 5′-CGTTCAAA GCTGCACACAGTC-3′ and probe 5′-6-carboxyfluorescein [FAM]-CAAAGC CCGCTCCTACCTGCAATATCA-6-carboxytetramethylrhodame [TAMRA]-3′) or the BHRF1-encoding gene segment (primers 5′-CGTGTGCATGGAAAT GGTACC-3′ and 5′-CGAAAGGCGGAGAGGTGTT-3′ and probe 5′-VIC-TGC ATCCTGTGTTGGAGCTAGCAGCA-TAMRA-3′). Results were normalized to cellular DNA encoding the C-reactive protein (CRP) (primers 5′-CTTGACCAGC CTCTCTCATGC-3′ and 5′-TGCAGTCTTAGACCCCACCC-3′ and probe 5′-VIC-TTTGGCCAGACAGGTAAGGGCCACC-TAMRA-5′). Serial dilutions of the lymphoid cell lines Namalwa or IB4 that contain a stable copy number of integrated EBV genomes per cell (26) were used as a standard for these assays. The EBV TR number unique to each cell clone was determined, as previously described (45), by calculating molecular weight from Southern blots of BamHI-digested total cellular DNA hybridized with a [α-^{32}P]dCTP-labeled NJhet fragment of EBV DNA as a probe.

RNA analyses by Northern blots and RT-PCR. RNA was extracted from cells according to the RNA-STAT60 protocol (Tel-Test). For cDNA synthesis, 10 μg RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) using random hexamer primers. RQ reverse transcription-PCR (RQ RT-PCR) to determine LMP1 mRNA levels was performed using the TaqMan fluorogenic system (ABI Prism 7700 sequence detection system; PE Applied Biosystems). The comparative cycle threshold (C_T) method was used to assess differences in LMP1 transcript levels, with C_T values determined by automated threshold analysis. All samples were run in duplicate, together with reactions to quantify expression of an internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to normalize for any differences in the amount of total RNA added. Template-negative and RT-negative reactions served as controls. The primers to quantify LMP1 mRNA in CCL20.2/B95-8 cells were 5′-AGACAAGTAAGCACCCGAAGAT-3′ and 5′-GCCCTTTGTATAC TCCTACTGATG-3′, which frame the exon1/2 splice junction present in both 2.8- and 3.5-kb messages, and the probe was 5′-FAM-CCCTCCTGCTCATCG CTCTCTGGA-TAMRA-3′. Primers to measure LMP1 mRNA in CCL185/Akata were 5′-AAGGCCAAAAGCTGCCAGAT-3′ and 5′-AATTTGCACGG ACAGGCATT-3′, which span the exon 2/3 splice junction, and the probe was 5′-FAM-TCCAGAACCTAAGACAAGTAAGCAGCCAAAGAT-TAMRA-3′. Human GAPDH mRNA was quantified using the predeveloped TaqMan assay reagents (Applied Biosystems).

LMP1 promoter usage was determined by the size of transcript with Northern blotting or RT-PCR specific for the 3.5-kb mRNA. For Northern blotting, polyadenylated RNA was purified from total cellular RNA using the Oligotex kit (Qiagen) per the manufacturer's instructions. LMP1 transcript size was determined on blots hybridized to [α-^{32}P]dCTP-labeled LMP1 cDNA (pSG-LMP1; gift of C. Sample, Pennsylvania State University College of Medicine), using the Millennium RNA marker (Ambion). For RT-PCR of the 3.5-kb LMP1 transcript, LMP1 cDNA was generated with an LMP1-specific oligo, 5′-GATGAA CAGCACAATTCCAAG-3′. Subsequent amplification of LMP1 cDNA employed a 3′ primer recognizing both 2.8-kb and 3.5-kb transcripts and either of two 5′ primers: the first common to both and the second 5′ to the first and specific to sequences unique to the 3.5-kb message (Fig. 1A). Primers for amplification of the 3.5-kb message were 5′-CAAATTCCAGAGAGCGATGAG-3′ and 5′-CACTGCCTTCCATTTCCTG-3′ and for both the 2.8-kb and 3.5-kb transcripts were 5′-CAAATTCCAGAGAGCGATGAG-3′ and 5′-TAGGCCTT GCTCTCCTTCTCCT-3′, and the probe was 5′-TCCAAGTGGACAGAGAAG GTCT-3′.

Preparation of lysates and immunoblotting. Whole-cell lysates were prepared using Laemmli buffer (0.5 M Tris-HCl, 1% sodium dodecyl sulfate [SDS], bromophenol blue, 1 M sucrose). Lysates were boiled for 5 min and then stored at −80°C. Protein equivalent to 2×10^4 cells for each sample was separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels. Proteins were transferred to Immobilon P membranes (Amersham). Membranes were incubated in blocking solution for 1 h at room temperature and then in primary antibody to LMP1 (CS1-4; Dako) overnight at 4°C. Using horseradish peroxidase-linked secondary antibody, proteins were visualized by enhanced chemiluminescence (ECL Plus; Amersham). Tubulin was used as a loading control. Densitometry to quantify protein was performed using Quantity One software.

L1-TR-driven GFP-reporter constructs. L1-TR-driven GFP reporter plasmids were constructed on the pEGFP1 promoterless vector backbone (Clontech). The pEGFP1 vector was digested with SspI and SfiI to remove the SV40 promoter and origin, preventing any read-through transcripts or enhancer effects on GFP expression from these distal elements (pEGFP1Δ). The Akata bacterial artificial chromosome 52 (AkBAC52; gift from Lindsey Hutt-Fletcher) was utilized to obtain a fused 10-TR DNA fragment. A BamHI-SalI TR-containing fragment was cloned into BamHI-HindIII sites in pBeloBac11 (New England Biolabs) by linker tailing. To generate plasmids containing a smaller number of TR, 10TR/pBeloBac11 was partially digested with EagI, which cuts once per TR. Partially digested DNAs were religated. Clones were screened by restriction digest, and a plasmid carrying 2 TRs was identified. To generate constructs with a TR number greater than 10, the 10TR/pBeloBac11 plasmid was linearized with AgeI and partially digested with EagI. DNA fragments of either 5 kb or greater than 15 kb in size were selected for isolation by gel purification and ligated at a 2:1 molar ratio. Resultant clones were screened by restriction enzyme digestion, and a clone carrying 17TR was identified. Next, the EcoRI-BssHII TR-containing DNA was cloned into the EcoRI-BamHI sites in pEGFP1Δ by linker tailing. Constructs were named 2TRGFP and 17TRGFP. Finally, the variations in plasmid size between the 2TR and 17TR reporter constructs were equalized by inserting a 7.7-kb EagI-SnaBI DNA fragment from lambda phage by blunt end ligation into HindIII of 2TRGFP (2TRλGFP).

Uninfected carcinoma cell lines CCL20.2 and CCL185 were transiently transfected with 2TRλGFP reporter, 17TRGFP reporter, or promoterless GFP reporter plasmids using Lipofectamine 2000 (Invitrogen) or Lipofectamine LTX (Invitrogen). Transfection efficiencies were calculated by RQ PCR for GFP DNA and normalized to values for the cellular gene CRP. GFP expression was documented by light microscopy, quantified by flow cytometric analysis, or determined by RQ RT-PCR for EGFP mRNA (primers 5′-GCAGTGCTTCAGC CGCTAC-3′ and 5′-GGGCATGGCGGACTTC-3′; probe 5′-FAM-CCGAC-CACATGAAGCAGCACGACTT-TAMRA-3′). EGFP mRNA values were normalized to cellular GAPDH mRNA. Serial dilutions of DNA and mRNA from vector-transfected cells were used to generate a standard curve.

Cell growth assays. Population doublings were determined from cells seeded into 6-well plates at 10^4 cells/well in the presence of 400 μg/ml G-418. At 24 and 48 h prior to reaching confluence, cells were trypsinized, a viable cell count was determined by trypan blue exclusion, and population doubling was calculated. Values were obtained based on four independent experiments.

Tetrazolium salt (MTS) cell proliferation assays were performed using the Cell Titer 96 aqueous one-solution cell proliferation assay (Promega) according to the manufacturer's instructions. Cells were seeded in triplicate in 6-well plates at 5×10^4 cells/well in 2 ml DMEM. MTS reagent was added at 24, 36, and 48 h postseeding and incubated for 4 h, and absorbance read at 490 nm. Colorimetric values plotted were subtracted from blanks.

Relative rates of DNA synthesis in cell clones were quantified by 5-bromo-2′-deoxyuridine (BrdU) incorporation assays using the *in situ* cell proliferation kit FLUOS (Roche) per the manufacturer's instructions. Cells were either seeded in replicate T-75 flasks at 5×10^5 cells per flask or onto 8-well chamber slides (Nunc) at 5×10^3 cells/well. At the time points indicated in the legend for Fig. 4, BrdU labeling reagent was added for 1 h. Cells were washed with PBS, fixed in a 70% EtOH/50 mM glycine solution, and stained with anti-BrdU FLUOS-conjugated antibody. Incorporated BrdU was visualized by light microscopy or quantified by flow cytometric analysis in trypsinized cells counterstained with propidium iodide.

Competitive clonal outgrowth assays were performed as previously described (42). Briefly, cellular clones distinguishable by the molecular size of EBV-fused termini were combined in mixed culture, passaged every 48 h, and assessed after each passage for the emergence of a predominant cell clone as determined by changes in relative intensity of TR bands by Southern blotting.

RESULTS

LMP1 expression varies inversely to TR number. To determine whether TR number affects LMP1 expression, we chose to examine infection of epithelial cells which, unlike B cells, express in approximately equal abundance both the 3.5- and 2.8-kb mRNAs (initiated from L1-TR and ED-L1 promoters, respectively) (47). Because coexpression of a second EBV oncoprotein LMP2A can indirectly modulate LMP1 levels (13, 53), the B95-8 recombinant EBfaV-GFP (51), in which LMP2A expression is blocked by insertion of an EGFP-Neo^r resistance cassette (LMP2$^-$ GFP$^+$), was selected for most of these studies. Moreover, in the prototype B95-8 strain, the region encoding most of the BART microRNAs capable of

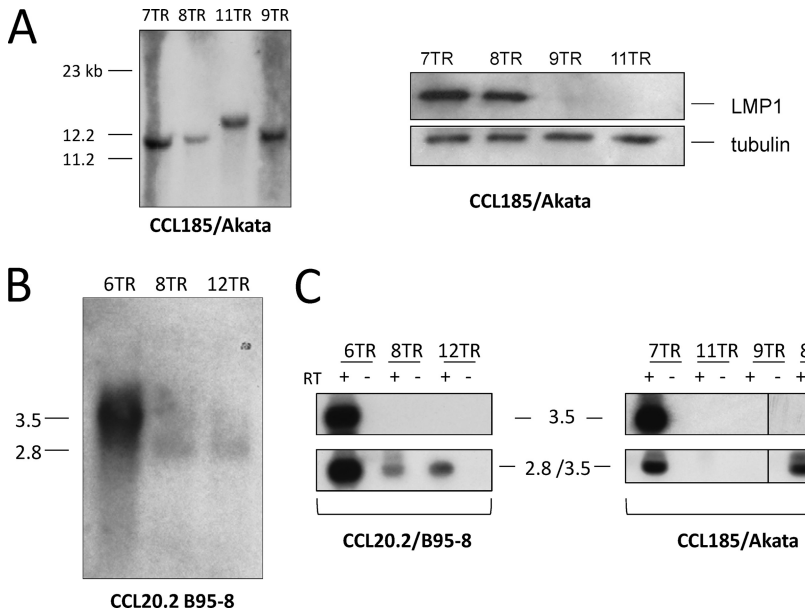

FIG. 2. Differential LMP1 promoter usage in epithelial cell culture linked to TR number. (A) Inverse correlation of LMP1 expression to terminal repeat number in CCL185/Akata. Southern blot (left) of BamHI-digested DNA from four infected epithelial cell clones probed for EBV terminal repeat sequences, with the TR number being calculated as indicated in the legend for Fig. 1. Molecular size markers are indicated on the left. Immunoblot (right) of LMP1 protein in each of four CCL185 clones. (B) Northern blot for LMP1 mRNA in CCL20.2/B95-8 clones. The 3.5-kb mRNA initiating from L1-TR promoter predominates in 6TR clone only. The sizes of the molecular weight markers (in thousands) are indicated on the left. (C) Increased L1-TR promoter activity at lower TR reiterations as determined by RT-PCR in two cell lines. PCR primers for amplification of cDNA positioned as shown in Fig. 1A. Top panels show amplification product specific for 3.5-kb transcript only, whereas bottom panels indicate PCR product derived from sequences common to both 2.8-kb and 3.5-kb transcripts.

inhibiting LMP1 translation is also deleted (37), minimizing a second potentially confounding factor for these studies.

Of 24 CCL20.2/B95-8 epithelial cell clones screened postinfection, 12 contained only the EBfaV-GFP recombinant without wild-type B95-8. Three were selected for study based on comparable episomal copy numbers of 35, 25, and 31 and divergent TR counts of 12, 8, and 6, respectively (Fig. 1B). We selected clones with comparable episomal copy numbers, despite ample evidence from past studies showing that EBV protein levels do not correlate with those of template DNA (33, 40, 52). All clones were negative for LMP2 and EBNA2 mRNA. By RQ RT-PCR, the level of LMP1 transcript was inversely proportional to the number of TR units (Fig. 1C), differences which were reflected in protein levels (Fig. 1D). Relative to the 12TR clone, the 8TR and 6TR clones had an increase of LMP1 protein of 2.5- and 7.6-fold, respectively, as determined by densitometric analysis. Because LMP1 levels in B cells have been reported to vary naturally up to 100-fold in individual cells of a clonal population (33), we examined 5 randomly selected subclones from each of the 6TR, 8TR, and 12TR clonal populations for consistency of mRNA expression levels. In contrast to what has been found with B cells, the levels of LMP1 expressed in each set of subclones of CCL20.2/B95-8 epithelial cells were uniform (data not shown). These data indicate that TR number plays a functional role in modulating LMP1 expression.

To assess whether the inverse correlation between LMP1 expression and TR reiteration was influenced by the viral strain or cell line used, we infected a second epithelial cell line, CCL185, with a recombinant Akata strain in which the neomycin resistance cassette had been inserted in the BDLF3 open reading frame (Fig. 1A) (4). CCL185/Akata clones with 11TR, 9TR, 8TR, and 7TR (Fig. 2A) contained 14, 15, 4, and 6 episomes, respectively. Clones were EBNA2 negative but LMP2A positive by RT-PCR. Despite the potentially confounding influence of LMP2A and BART miRNAs on LMP1 expression, results with CCL185/Akata confirmed the outcome observed with CCL20.2/B95-8. The two clones with the least TRs expressed the most LMP1, whereas the two clones with the most TRs expressed none (Fig. 2A).

Activity of L1-TR promoter predominates at low TR number. To determine whether one or both promoters were affected by TR count, mRNA was analyzed by Northern blotting for the 2.8-kb versus 3.5-kb message (Fig. 2B). In CCL20.2/B95-8 cells, the 2.8-kb message originating from the ED-L1 promoter was detected with 12TR and 8TR cell clones. In contrast, the 3.5-kb message predominated in the 6TR clone that expressed the highest levels of LMP1. To confirm further that L1-TR activity was affected by TR reiteration, an RT-PCR strategy specific for the 3.5-kb transcript was applied to both CCL20.2/B95-8 and CCL185/Akata clone sets (Fig. 2C). RT-PCR confirmed results from Northern blots for the B95-8-infected CCL20.2 clones, with only the 6TR clone yielding product for the larger message initiating at L1-TR. Likewise, in the two Akata-infected CCL185 clones expressing LMP1, only the 7TR clone utilized the L1-TR promoter (Fig. 2C).

Because LMP1 expressed from recombinant EBV might be subject to the modulating effects of other viral genes and possibly a position-dependent effect of drug resistance cassettes (9, 12), we constructed GFP reporter plasmids in which GFP

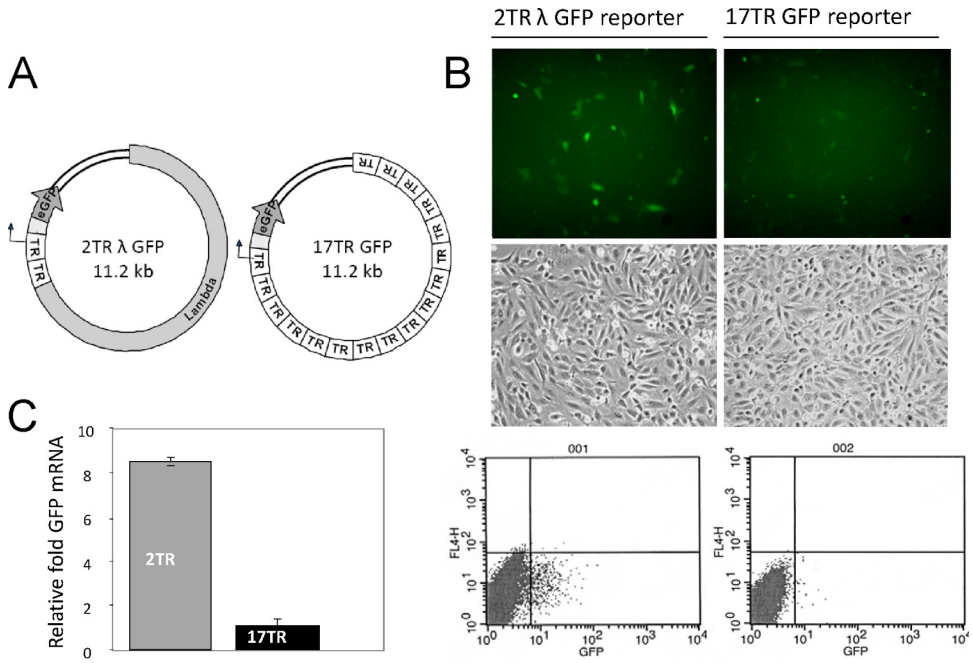

FIG. 3. TR reiteration influences GFP production from a L1-TR promoter-driven reporter construct in CCL20.2 epithelial cells. (A) Schematic of GFP reporters of equivalent size, one with 2TRs and a 7.6-kb λ phage DNA stuffer and the second with 17TRs. (B) Fluorescence microscopy of epithelial cells 24 h posttransfection with GFP reporter constructs (top); cell density as shown by phase-contrast microscopy (middle); GFP expression in respective cell populations by flow cytometric analysis (bottom). (C) GFP mRNA relative to transfected plasmid copy number as determined by RQ RT-PCR. Values shown are from three independent experiments, with error bars representing the SEM.

expression was driven by L1-TR in the context of variable TR numbers. Plasmids with 2TR versus 17TR inserted 5′ to the GFP gene (Fig. 3A), but made equivalent in size by insertion of λ-phage stuffer DNA, were transiently transfected into EBV-negative CCL20.2 epithelial cells, and GFP expression was determined. As shown by fluorescent microscopy, cell cultures transfected with 2TRλGFP constructs contained more positive cells with higher levels of fluorescence than those transfected with the 17TRGFP construct, despite similar efficiencies of transfection, as determined by RQ PCR for GFP DNA (Fig. 3B). These findings were corroborated by flow cytometric analysis (Fig. 3B, bottom). When evaluated by RQ RT-PCR, the 2TRλGFP plasmid expressed eightfold greater GFP mRNA than did the 17TRGFP plasmid (Fig. 3C). The outcome in EBV-negative CCL185 cells was essentially identical (data not shown).

Growth and morphological alterations in TR-defined clonal populations. Forced expression of LMP1 in transfection assays has been reported to produce striking alterations in epithelial cell morphology, characterized by a loss of the original cuboidal appearance and the development of a more fusiform morphology (18, 32, 36, 43). Surprisingly, when expressed here in the physiologically relevant context of virus infection, LMP1 conferred on the three clones of the CCL20.2 carcinoma cell line a graduated change in phenotype that paralleled stepwise increases in LMP1 expression levels (Fig. 4A). Clones lost the cobblestone appearance of the uninfected parental cells and acquired a long spindle-like morphology which became increasingly exaggerated at the physiological high end of LMP1 expression represented by the 6TR clone. Likewise, increased vacuolization of cells was evident at elevated levels of LMP1 (Fig. 4A).

In B lymphocytes, as little as a twofold increase in LMP1 expression is sufficient to change a proliferative phenotype to a cytostatic one (29). To determine the effect of divergent levels seen here on short-term growth characteristics of epithelial cell clones, CCL20.2/B95-8 population doublings were assessed by direct cell counts after seeding at low density (Fig. 4B). At 48 h and prior to achieving cell confluence, the 8TR clone with intermediate levels of LMP1 was most proliferative. Less growth was observed with clones with the least (12TR) and most (6TR) LMP1 expression, the latter being relatively cytostatic (Fig. 4B). No differences in viability were observed by trypan blue exclusion.

As confirmation, the MTS assay was performed to measure the metabolic activity of mitochondrial dehydrogenase, which serves as an indicator of cell number and proliferation (Fig. 4C). Intermediate expression of LMP1 in the 8TR clone correlated with the highest levels of MTS activity, with either extreme of LMP1 in the 12TR and 6TR clones linked to reduced MTS activity (Fig. 4C). Likewise, when incorporation of BrdU was used as an indicator of cell proliferation, CCL20.2/B95-8 clones expressing high (6TR) and low (12TR) levels of LMP1 showed a reduction in the number of cells incorporating BrdU relative to the clone expressing intermediate (8TR) levels of LMP1 (Fig. 4D). In contrast to the divergent growth profiles manifest by the three infected clones, growth properties of randomly chosen EBV-negative CCL20.2 clones were identical when tested in short-term growth assays

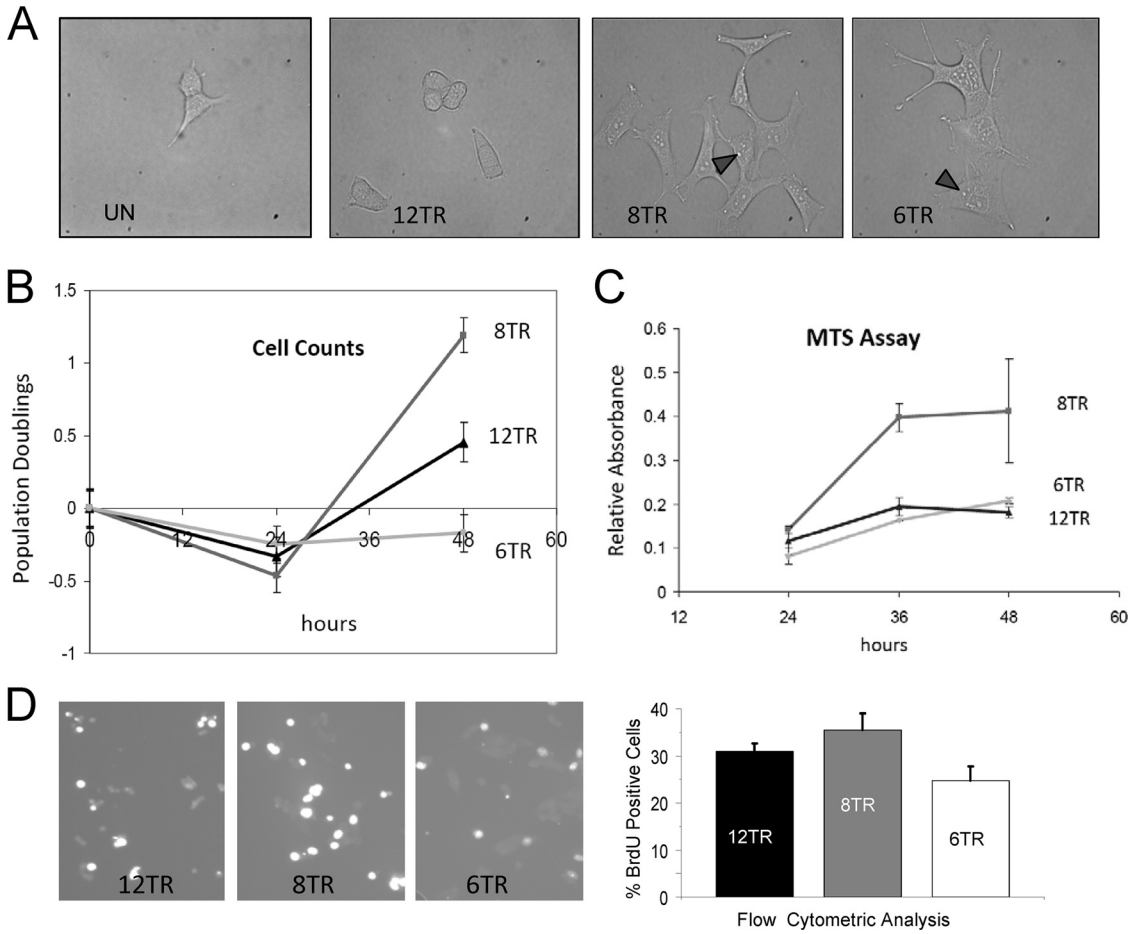

FIG. 4. Morphological and growth phenotypes of TR-defined epithelial cell clones. (A) CCL20.2/B95-8 clones examined 24 h post seeding at 10^4 cells/ml. From left to right, uninfected cells (UN) followed by infected cells with least (12TR) to most (6TR) LMP1 expression. Arrowheads indicate vacuolization. (B) Population doublings in short-term growth assay of CCL20.2/B95-8 clones. Cells were seeded at 10^4 cells/ml and then counted at indicated times prior to reaching confluence. Each time point represents the mean of 4 independent experiments, the error bars indicating SEM. (C) Proliferation of CCL20.2/B95-8 clones as indicated by metabolic conversion of dye in MTS assay. Colorimetric values plotted were readings minus values obtained from blanks. Error bars indicate the SEM of values from 3 independent experiments. (D) Relative rates of DNA synthesis in CCL20.2/B95-8 cell clones as quantified by BrdU incorporation. At left, BrdU uptake in 1 h by cells seeded 24 h earlier, as visualized by fluorescent microscopy after staining with anti-BrdU FLUOS-conjugated antibody. At right, percent of BrdU-positive cells as measured by flow cytometry. Cells were treated for 1 h with BrdU 24 h after seeding and stained as described above. Error bars denote SEM from 3 independent experiments.

(data not shown), making underlying clonal variation of the host cell an unlikely explanation for results seen.

To determine if short-term growth results were predictive of the ultimate emergence over time of a predominant EBV-infected clone from a mixed cell population, the three CCL20.2/B95-8 clones were combined at various ratios and passaged every 48 h, a time frame consistent with the proliferation assays described above (Fig. 5A). The relative intensity on Southern blots of each TR band at any given passage was determined by densitometry as a measure of the changing proportions of the three cell clones. In the result shown, the starting cell population was weighed heavily in favor of the 6TR clone, with the slowest growth rate in short-term assays. By passage 15, the makeup of the bulk population had changed dramatically. There was an overall reduction in total percentage of cells derived from the 6TR clone relative to an increasingly predominant 8TR clone (Fig. 5B), corroborating short-term growth measurements.

DISCUSSION

This study adds to a growing number of investigations that suggest heterogeneity intrinsic to repetitive sequences situated throughout the EBV genome affects functional efficiency of virus (1, 41, 42, 55). The effect of TR number on LMP1 levels adds yet another layer of complexity to the regulation of LMP1 expression and opens the possibility that the end-to-end length of fused TRs drives clonal selection consequent to the opposing growth advantages and disadvantages of this oncoprotein. In single-cell clones of carcinoma cell lines infected with EBV, LMP1 expression was inversely proportional to TR number, leading to relatively cytostatic as well as proliferative growth

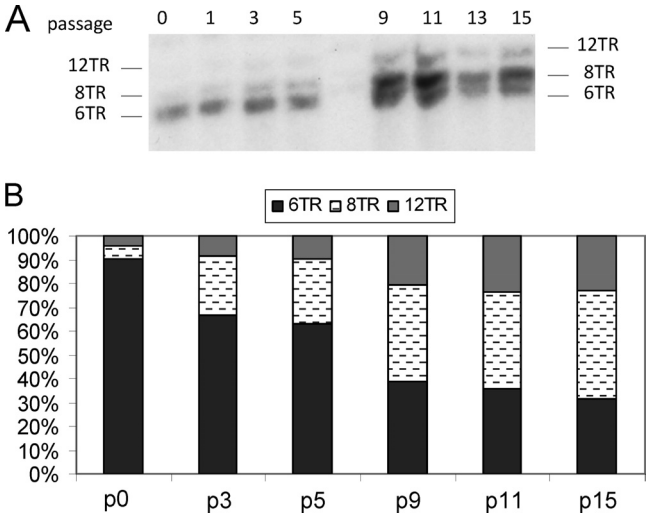

FIG. 5. Cytostatic versus proliferative phenotypes of the three CCL20.2/B95-8 epithelial cell clones in mixed culture, with emergence of 8TR clone as predominant. (A) Southern blot hybridized to EBV terminus-specific probe, with each lane representing a sequential passage as indicated by number. 12TR, 8TR, and 6TR clones were mixed disproportionately for seeding at time zero to favor slower-growing 6TR clone, and then passaged every 48 h × 15. Results shown are representative of 3 independent experiments. (B) Proportion of total cell population represented by each clone at indicated passage, as determined by densitometry of bands within single lane.

phenotypes in respective cell clones. An unexpected finding was the differential promoter usage seen with shifting TR numbers, with there being an apparent preference for L1-TR at lower reiterations of TRs for which LMP1 expression was greatest. Comparatively higher expression from L1-TR is not surprising with epithelial cell systems, since robust activity of ED-L1 requires EBNA2, which is lacking in epithelial cells. EBNA2-independent activation of ED-L1 by cellular factors is relatively modest (34, 44).

Although infectious virus provides gene expression levels that are physiologically relevant, one caveat should be raised pertaining to our use of the B95-8 recombinant EBfaV-GFP in these experiments. The GFP cassette, situated so as to disrupt the LMP2A gene, was placed upstream of the LMP1 promoters but in an opposite orientation to LMP1 transcription to obviate a downstream effect. However, the TRs are sandwiched between these two elements (Fig. 1A), and the variable size of fused TR units in each CCL20.2/B95-8 clone raises the possibility that differential use of L1-TR reflects a position-dependent effect of the GFP cassette relative to that promoter (9, 12). Two findings largely negate this concern. First, ED-L1 activity was again supplanted by L1-TR at a low TR reiteration in the second virus strain, Akata, where the drug-resistant cassette was placed some 38 kb downstream of the LMP1 gene and equidistant from its promoters in all 4 cell clones. Second, when TR sequences containing only L1-TR were inserted at increasing reiterations 5′ to the GFP gene in a promoterless reporter construct, the greatest GFP expression occurred at the minimal TR number in both carcinoma cell lines transiently transfected with the constructs.

The reason for the relative inactivity of L1-TR at higher TR reiterations is as yet unclear. However, the absence of other viral factors in the reporter construct supports an instrumental role for TR copy number *per se*. Though transcription is initiated only from within the first TR (47), each adjacent repetitive unit increases the number of competitive binding sites for cellularly derived transcription factors, such as Sp1, Sp3, and STAT3, thought to interact cooperatively. Aside from the Sp1 binding sites contained in the GC box of this TATA-less promoter that are key to L1-TR activity (47, 54), additional binding sites exist in the GC-rich TRs that bind Sp1 more efficiently than classical Sp1 sites (50). While thought to put EBV at an advantage with respect to attracting Sp1 away from the cellular genome (50), their higher binding affinities might also outcompete L1-TR for uncommitted Sp1 molecules as the abundance of binding sites increases, effectively silencing that promoter. Alternatively, the location of L1-TR in the first TR may make it more vulnerable to methylation at higher reiterations of CpG-rich TRs. However, silencing by mechanisms other than methylation appears likely, given the results from transient transfection of two nonreplicating L1-TR-driven GFP constructs in which methylation is not in play.

Cellular phenotypic changes observed in this study are consistent with those previously described as accompanying increased levels of LMP1 expression (18, 32, 36, 43). In short-term proliferation assays, the 8TR CCL20.2/B95-8 clone expressing intermediate levels of LMP1 grew fastest, whereas the 12TR clone with the least and 6TR clone with the greatest LMP1 grew slower, if at all. Such strikingly diverse outcomes in clonal cell growth with the relatively small differences in LMP1 expression illustrated here suggest one means by which a rapid transition from polyclonal to monoclonal infection could occur based on optimal TR number, even should infection occur as a late event after tumor initiation. Accordingly, in long-term culture, the 8TR clone emerged as predominant in mixed culture.

Retarded growth accompanying the high LMP1 levels seen with L1-TR-initiated transcription in the 6TR clone does not preclude L1-TR activation in proliferative states, however, as evidenced by utilization of that promoter in Hodgkin's lymphoma and nasopharyngeal carcinoma (8), two tumors infiltrated by a large T-cell component. The incremental increase in vacuolization of epithelial cell clones apparent with increasing LMP1 levels (Fig. 4A) is consistent with formation of multivesicular bodies involved in the extracellular release of LMP1-containing exosomes that occurs in epithelial cells as well as lymphocytes (6, 19, 30). In EBV-infected B cells, LMP1 secretion via exosomes exerts a direct immunosuppressive effect on T-cell proliferation and NK-cell cytotoxicity, suggesting the likelihood of a similar effect on tumor-infiltrating lymphocytes (14).

Functional multivesicular bodies are also thought to be required intermediaries of efficient autophagic degradation of cytoplasmic proteins (17). Autophagy is triggered by a variety of extracellular signals, to include the endoplasmic reticulum (ER) stress-activated unfolded protein response (UPR). Both ED-L1 and L1-TR are downstream of signaling pathways involved in the UPR, each promoter containing distinct ER stress response elements. PERK/ATF4 signaling activates ED-L1 in B cells (34), whereas the inositol-requiring kinase 1 (IRE1)-spliced UPR protein XBP-1 triggers L1-TR in nasopharyngeal carcinoma-derived epithelial cells (27). Notably in

B cells, EBNA2 induction of LMP1 leads to PERK activation and initiation of an autocatylytic loop of LMP1 self-regulation to circumvent the cytostatic effects of overexpression (34). Finally, the detrimental growth effects of enhanced transcription from L1-TR observed in our *in vitro* assay might be offset posttranscriptionally by the activity of EBV BART miRNAs expressed at high levels in NPC (37). Inhibition of LMP1 translation by BART microRNAs has been posited as a likely explanation for the disparity between mRNA and protein levels often noted in NPC (37).

Perhaps the greater import of TR heterogeneity as a factor governing LMP1 expression lies ironically in the very homogeneity of TRs in EBV-linked malignancies, in which they serve to define tumor clonality (45). If every tumor cell contains episomes of a uniform TR number, the assumption is that virus was present prior to clonal expansion and thereby contributed to tumor initiation. There is increasing evidence, however, that EBV infection may also occur as a late event in multistep carcinogenesis, contributing in these cases to tumor progression (38, 42). Our infection of carcinoma cells further validates such a view by providing evidence for clonal emergence based on a TR number best suited for LMP1 optimization, negating the supposition that late infections of tumors would by necessity be polyclonal. Finally, our findings amply accommodate lytic replication as a component of the malignant process. EBV reactivation and reinfection prior to clinical onset of disease would serve to generate episomes of sufficient diversity in TRs (48) to ensure LMP-1-modulated cell growth and survival in any given tumor cell microenvironment.

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REFERENCES

1. **Ali, A. K. M., S. Saito, S. Shibata, K. Takada, and T. Kanda.** 1 July 2009. Distinctive effects of the Epstein-Barr virus family of repeats on viral latent gene promoter activity and B-lymphocyte transformation. J. Virol. doi: 10.1128/JVI.01979-08.
2. **Baer, R. J., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. F. Hatfill, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. C. Barrell.** 1984. DNA sequences and expression of the B95-8 Epstein-Barr virus genome. Nature **310:**207–211.
3. **Blackwood, E. M., and J. T. Kadonaga.** 1998. Going the distance: a current review of enhancer action. Science **281:**60–63.
4. **Borza, C. M., and L. M. Hutt-Fletcher.** 1998. Epstein-Barr virus recombinant lacking expression of glycoprotein gp150 infects B cells normally but is enhanced for infection of epithelial cells. J. Virol. **72:**7577–7582.
5. **Brown, N. A., C.-R Liu, Y.-F. Wang, and C. R. Garcia.** 1988. B-cell lymphoproliferation and lymphomagenesis are associated with clonotypic intracellular terminal regions of the Epstein-Barr virus. J. Virol. **62:**962–969.
6. **Ceccarelli, S., V. Visco, S. Raffa, N. Wakisaka, J. S. Pagano, and M. R. Torrisi.** 2007. Epstein-Barr virus latent membrane protein 1 promotes concentration in multivesicular bodies of fibroblast growth factor 2 and its release through exosomes. Int. J. Cancer. **121:**1494–1506.
7. **Chang, M.-H., C.-K. Ng, Y.-J. Lin, C.-L. Liang, P.-J. Chung, M.-L. Chen, Y.-S. Tyan, C.-Y. Hsu, C.-H. Shu, and Y.-S. Chang.** 1997. Identification of a promoter for the latent membrane protein 1 gene of Epstein-Barr virus that is specifically activated in human epithelial cells. DNA Cell Biol. **16:**829–837.
8. **Chen, H., J. M. Lee, Y. Zong, M. Borowitz, M. H. Ng, R. F. Ambinder, and S. D. Hayward.** 2001. Linkage between STAT regulation and Epstein-Barr virus gene expression. J. Virol. **75:**2929–2937.
9. **Clambey, E. T., H. W. Virgin IV, and S. H. Speck.** 2000. Disruption of the murine gammaherpesvirus68 M1 open reading frame leads to enhanced reactivation from latency. J. Virol. **74:**1973–1984.
10. **Coffin, W. F., III, T. R. Geiger, and J. M. Martin.** 2003. Transmembrane domains 1 and 2 of the latent membrane protein 1 of Epstein-Barr virus contains a lipid raft targeting signal and plays a critical role in cytostasis. J. Virol. **77:**3749–3758.
11. **Cuomo, L., T. Ramquist, P. Trivedi, F. Wang, G. Klein, and M. G. Masucci.** 1992. Expression of the Epstein-Barr virus (EBV)-encoded membrane protein LMP1 impairs the *in vitro* growth, clonability and tumorigenicity of an EBV-negative Burkitt lymphoma line. Int. J. Cancer **51:**949–955.
12. **D'Aiuto, L. D., R. de Marco, E. Nwanegbo, A. Rizzo, J. R. Chaillet, A. Montecalvo, M. T. Lotze, and A. Gambotto.** 2006. Evidence of the capability of the CMV enhancer to activate in *trans* gene expression in mammalian cells. DNA Cell Biol. **25:**171–180.
13. **Dawson, C. W., J. H. George, S. M. S. Blake, R. Longnecker, and L. S. Young.** 2001. The Epstein-Barr virus encoded latent membrane protein 2A augments signaling from latent membrane protein 1. Virology **289:**192–207.
14. **Dukers, D. F., P. Meij, M. B. H. J. Vervoort, W. Vos, R. J. Scheper, C. J. L. M. Meijer, E. Bloemena, and J. M. Middeldorp.** 2000. Direct immunosuppressive effects of EBV-encoded latent membrane protein 1. J. Immunology **165:**663–670.
15. **Eliopoulos, A. G., C. W. Dawson, G. Mosialos, J. E. Floettmann, M. Rowe, R. J. Armitage, J. Dawson, J. M. Zapata, D. J. Kerr, M. J. Wakelam, J. C. Reed, E. Kieff, and L. S. Young.** 1996. CD40-induced growth inhibition in epithelial cells is mimicked by Epstein-Barr virus-encoded LMP1: involvement of TRAF3 as a common mediator. Oncogene **13:**2243–2254.
16. **Everly, D. N., Jr., B. A. Mainou, and N. Raab-Traub.** 2004. Induction of Id1 and Id3 by latent membrane protein 1 of Epstein-Barr virus and regulation of p27/Kip and cyclin-dependent kinase 2 in rodent fibroblast transformation. J. Virol. **78:**13470–13478.
17. **Fader, C. M., and M. I. Colombo.** 2009. Autophagy and multivesicular bodies: two closely related partners. Cell Death Differ. **16:**70–78.
18. **Fahraeus, R., L. Rymo, J. S. Rhim, and G. Klein.** 1990. Morphological transformation of human keratinocytes expressing the LMP gene of Epstein-Barr virus. Nature **345:**447–449.
19. **Flanagan, J., J. Middeldorp, and T. Sculley.** 2003. Localization of the Epstein-Barr virus protein LMP1 to exosomes. J. Gen. Virology **84:**1871–1879.
20. **Floettmann, J. E., K. Ward, A. B. Rickinson, and M. Rowe.** 1996. Cytostatic effect of Epstein-Barr virus latent membrane protein-1 analyzed using tetracycline-regulated expression in B cell lines. Virology **223:**29–40.
21. **Gahn, T. A., and B. Sugden.** 1995. An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. J. Virol. **69:**2633–2636.
22. **Gilligan, K., H. Sato, P. Rajadurai, P. Busson, L. Young, A. Rickinson, T. Tursz, and N. Raab-Traub.** 1990. Novel transcription from the Epstein-Barr virus terminal EcoRI fragment, DIJhet, in a nasopharyngeal carcinoma. J. Virol. **64:**4948–4956.
23. **Gires, O., F. Kohlhuber, E. Kilger, M. Baumann, A. Kieser, C. Kaiser, R. Zeidler, B. Scheffer, M. Ueffing, and W. Hammerschmidt.** 1999. Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins. EMBO J. **18:**3064–3073.
24. **Hammarskjöld, M. L., and M. C. Simurda.** 1992. Epstein-Barr virus latent membrane protein transactivates the human immunodeficiency virus type 1 long terminal repeat through induction of NF-kappa B activity. J. Virol. **66:**6496–6501.
25. **Hammerschmidt, W., B. Sugden, and V. R. Baichwal.** 1989. The transforming domain alone of the latent membrane protein of Epstein-Barr virus is toxic to cells when expressed at high levels. J. Virol. **63:**2469–2475.
26. **Henderson, A., S. Ripley, M. Heller, and E. Kieff.** 1983. Chromosome site for Epstein-Barr virus DNA in a Burkitt tumor cell line and in lymphocytes growth-transformed *in vitro.* Proc. Natl. Acad. Sci. U. S. A. **80:**1987–1991.
27. **Hsiao, J. R., K. C. Chang, C. W. Chen, S. Y. Wu, I. J. Su, M. C. Hsu, Y. T. Jin, S. T. Tsai, K. Takada, and Y. Chang.** 2009. Endoplasmic reticulum stress triggers XBP-1-mediated up-regulation of an EBV oncoprotein in nasopharyngeal carcinoma. Cancer Res. **69:**4461–4467.
28. **Johannsen, E., E. Koh, G. Mosialos, X. Tong, E. Kieff, and S. R. Grossman.** 1995. Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by Jκ and PU.1. J. Virol. **69:**253–262.
29. **Kaykas, A., and B. Sugden.** 2000. The amino-terminus and membrane-spanning domains of LMP-1 inhibit cell proliferation. Oncogene **19:**1400–1410.
30. **Keryer-Bibens, C., C. Pioche-Durieu, C. Villemant, S. Souquere, N. Nishi, M. Hirashima, J. Middeldorp, and P. Busson.** 2006. Exosomes released by EBV-infected nasopharyngeal carcinoma cells convey the viral latent membrane protein 1 and the immunomodulatory protein galectin 9. BMC Cancer **6:**283–290.
31. **Kieser, A., E. Kilger, O. Gires, M. Ueffing, W. Kolch, and W. Hammerschmidt.** 1997. Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade. EMBO J. **16:**6478–6485.
32. **Kim, K.-R, T. Yoshizaki, H. Miyamori, K. Hasegawa, T. Horikawa, M. Furukawa, S. Harada, M. Seiki, and H. Sato.** 2000. Transformation of Madin-Darby canine kidney (MDCK) epithelial cells by Epstein-Barr virus latent membrane protein 1 (LMP1) induces expression of Ets1 and invasive growth. Oncogene **19:**1764–1771.
33. **Lam, N., M. L. Sandberg, and B. Sugden.** 2004. High physiological levels of

LMP1 result in phosphorylation of eIF2α in Epstein-Barr virus-infected cells. J. Virol. **78:**1657–1664.
34. **Lee, D. Y., and B. Sugden.** 2008. The LMP1 oncogene of EBV activates PERK and the unfolded protein response to drive its own synthesis. Blood **111:**2280–2289.
35. **Li, H. M., Z. H. Zhuang, Q. Wang, J. C. S. Pang, X. H. Wang, H. L. Wong, H. C. Feng, D. Y. Jin, M. T. Ling, Y. C. Wong, A. G. Eliopoulos, L. S. Young, D. P. Huang, and S. W. Tsao.** 2004. Epstein-Barr virus latent membrane protein 1 (LMP1) upregulates Id1 expression in nasopharyngeal epithelial cells. Oncogene **23:**4488–4494.
36. **Lo, A. K. F., D. P. Huang, K. W. Lo, Y. L. Chui, H. M. Li, J. C. S. Pang, and S.-W Tsao.** 2004. Phenotypic alterations induced by the Hong Kong-prevalent Epstein-Barr virus-encoded LMP1 variant (2117-LMP1) in nasopharyngeal epithelial cells. Int. J. Cancer **109:**919–925.
37. **Lo, A. K. F., K. F. To, K. W. Lo, R. W. M. Lung, J. W. Y. Hui, G. Liao, and S. D. Hayward.** 2007. Modulation of LMP1 protein expression by EBV-encoded microRNAs. Proc. Natl. Acad. Sci. U. S. A. **104:**16164–16169.
38. **Lo, K. W., and D. P. Huang.** 2002. Genetic and epigenetic changes in nasopharyngeal carcinoma. Semin. Cancer Biol. **12:**451–462.
39. **Longnecker, R., C. L. Miller, B. Tomkinson, X.-Q. Miao, and E. Kieff.** 1993. Deletion of DNA encoding the first five transmembrane domains of Epstein-Barr virus latent membranes 2A and 2B. J. Virol. **67:**5068–5074.
40. **Metzenberg, S.** 1990. Levels of Epstein-Barr virus DNA in lymphoblastoid cell lines are correlated with frequencies of spontaneous lytic growth but not with levels of expression of EBNA1, EBNA2, or latent membrane protein. J. Virol. **64:**437–444.
41. **Moody, C. A., R. S. Scott, N. Amirghahari, C. A. Nathan, L. S. Young, C. W. Dawson, and J. W. Sixbey.** 2005. Modulation of the cell growth regulator mTOR by Epstein-Barr virus-encoded LMP2A. J. Virol. **79:**5499–5506.
42. **Moody, C. A., R. S. Scott, T. Su, and J. W. Sixbey.** 2003. Length of Epstein-Barr virus termini as a determinant of epithelial cell clonal emergence. J. Virol. **77:**8555–8561.
43. **Morris, M. A., C. W. Dawson, W. Wei, J. D. O'Neil, S. S. Stewart, J. Jia, A. I. Bell, L. S. Young, and J. R. Arrand.** 2008. Epstein-Barr virus-encoded LMP1 induces a hyperproliferative and inflammatory gene expression programme in cultured keratinocytes. J. Gen. Virol. **89:**2806–2820.
44. **Ning, S., A. M. Hahn, L. E. Huye, and J. S. Pagano.** 2003. Interferon regulatory factor 7 regulates expression of Epstein-Barr virus latent membrane protein 1: a regulatory circuit. J. Virol. **77:**9359–9368.
45. **Raab-Traub, N., and K. Flynn.** 1986. The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation. Cell **47:**883–889.
46. **Reisman, D., and B. Sugden.** 1986. Trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol. **6:**3838–3846.
47. **Sadler, R. H., and N. Raab-Traub.** 1995. The Epstein-Barr virus 3.5-kilobase latent membrane protein 1 mRNA initiates from a TATA-less promoter within the first terminal repeat. J. Virol. **69:**4577–4581.
48. **Sato, H., T. Takimoto, S. Tanaka, J. Tanaka, and N. Raab-Traub.** 1990. Concatameric replication of Epstein-Barr virus: structure of the termini in virus-producer and newly transformed cell lines. J. Virol. **64:**5295–5300.
49. **Sjöblom, A., W. Yang, L. Palmqvist, A. Jansson, and L. Rymo.** 1998. An ATF/CRE element mediates both EBNA2-dependent and EBNA2-independent activation of the Epstein-Barr virus LMP1 gene promoter. J. Virol. **72:**1365–1376.
50. **Spain, T. A., R. Sun, and G. Miller.** 1997. The locus of Epstein-Barr virus terminal repeat processing is bound with enhanced affinity by Sp1 and Sp3. Virology **237:**137–147.
51. **Speck, P., K. A. Kline, P. Cheresh, and R. Longnecker.** 1999. Epstein-Barr virus lacking latent membrane protein 2 immortalizes B cells with efficiency indistinguishable from that of wild-type virus. J. Gen. Virol. **80:**2193–2203.
52. **Sternås, L., T. Middleton, and B. Sugden.** 1990. The average number of molecules of Epstein-Barr nuclear antigen 1 per cell does not correlate with the average number of Epstein-Barr virus (EBV) DNA molecules per cell among different clones of EBV-immortalized cells. J. Virol. **64:**2407–2410.
53. **Stewart, S., C. W. Dawson, K. Takada, J. Curnow, C. A. Moody, J. W. Sixbey, and L. S. Young.** 2004. Epstein-Barr virus-encoded LMP2A regulates viral and cellular gene expression by modulation of the NF-κB transcription factor pathway. Proc. Natl. Acad. Sci. U. S. A. **101:**15730–15735.
54. **Tsai, C.-N., C.-M. Lee, C.-K. Chien, S.-C. Kuo, and Y.-S. Chang.** 1999. Additive effect of Sp1 and Sp3 in regulation of the ED-L1E promoter of the EBV LMP1 gene in human epithelial cells. Virology **261:**288–294.
55. **Xue, S.-A., M. D. Jones, Q.-L. Lu, J. M. Middeldorp, and B. E. Griffin.** 2003. Genetic diversity: frameshift mutations alter coding of a gene (Epstein-Barr virus LF3 gene) that contains multiple 102-base-pair direct sequence repeats. Mol. Cell. Biol. **23:**2192–2201.